17α-Hydroxyprogesterone, 4-Androstenedione, and Testosterone Profiled by Routine Stable Isotope Dilution/Gas Chromatography-Mass Spectrometry in Plasma of Children

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ABSTRACT

Using stable isotope dilution/gas chromatography-mass spectrometry (ID/GC-MS), a physicochemical method, we have profiled the plasma steroids 17α-hydroxyprogesterone, 4-androstenedione, and testosterone in normal children of various age groups. Comparison of our values with those obtained by direct immunologic assays and those using an extraction or purification step showed that immunoassays in general overestimate steroid concentrations. This was especially true for plasma samples in the neonatal period and was most expressed for the concentrations of 17α-hydroxyprogesterone. Our study demonstrated the applicability of ID/GC-MS to routine clinical steroid analysis. The application of ID/GC-MS is recommended whenever problems from matrix effects or cross-reactivity are likely to arise or suspicious results by immunoassays need to be rechecked. (*Pediatr Res* **38:** 76–80, 1995)

Abbreviation

ID/GC-MS, isotope dilution/gas chromatography-mass spectrometry

Plasma 17α-hydroxyprogesterone (17α-hydroxypregn-4-ene-3,20-dione), 4-androstenedione (androst-4-ene-3,17-dione), and testosterone (17β-hydroxyandrost-4-en-3-one) are important parameters for the diagnosis and monitoring of hyperandrogenic disorders, most importantly 21-hydroxylase deficiency (1). Currently, their routine determination is almost exclusively based on immunoassays. However, the reliability of steroid immunoassays was shown to be questionable when problems due to cross-reactivity or matrix effects are likely to arise (2–9).

To circumvent these problems, we have recently developed a method based on stable ID/GC-MS allowing the simultaneous determination of 17α-hydroxyprogesterone, 4-androstenedione, and testosterone (10). It was the purpose of this study to obtain first mass spectrometric data on the plasma concentrations of these key steroids of androgen metabolism in children of various age groups and to further demonstrate the applicability of ID/GC-MS to clinical plasma steroid analysis.

METHODS

Subjects. We have analyzed plasma samples from 45 female and 48 male subjects. Infants and children were admitted for minor, non-endocrine disorders. Informed consent was obtained from their parents. Samples from adults were donated by staff members. The samples were collected between 0800 and 1000 h. Furthermore, 20 umbilical cord blood specimens were collected from 10 female and 10 male full-term neonates born spontaneously after an uneventful pregnancy. All blood specimens were immediately centrifuged, and the plasma was stored at −20°C until assay.

ID/GC-MS. Plasma 17α-hydroxyprogesterone, 4-androstenedione, and testosterone were determined in a single profile according to our own ID/GC-MS procedure (10). Deuterium-labeled analogs of the steroids—17α-hydroxy-[11,11,12,12-$^2H_4$]progesterone, [7,7-$^2H_2$]androst-4-enedione, [16,16,17-$^2H_3$]testosterone—served as internal standards. Plasma (0.5 mL in male subjects, 0.5–2 mL in female subjects and children) was equilibrated with a cocktail containing the internal standards. After solvent extraction, the dried organic extracts were purified on Sephadex LH-20 minicolumns. Then, heptafluoro-butyric acid derivatives were prepared, and a 0.1 aliquot of a processed plasma extract was analyzed. Gas chromatography was carried out on an OV-1 fused silica column (Macherey-Nagel, FRG; 25 m × 0.15 mm; film thickness 0.1 μm) housed

in a DANI 6500 gas chromatograph. The gas chromatograph was directly interfaced to a Hewlett Packard 5970B mass selective detector operated in the selected ion monitoring mode. Quantitation was performed using the peak area ratios between the ion pairs of the analytes and their corresponding labeled analogs.

For the steroids studied, intra- and interassay coefficients of variation were between 3.5–4.1% and 3.8–3.9%, respectively. Sensitivity was lowest for testosterone with a signal to noise ratio of 2.4 for 10 pg, and highest for 17α-hydroxyprogesterone with a signal to noise ratio of 5.3 for 10 pg. Accuracy was determined by spiking plasma with known amounts of steroids. The agreement between the values found and the amounts added was excellent with a relative error less than 7.5%. Standard plots were linear: 17α-hydroxyprogesterone, $y = 1.88x + 0.18$, $r = 0.999$; 4-androstenedione, $y = 1.37x - 0.24$, $r = 0.997$; testosterone, $y = 1.63x - 0.35$, $r = 0.999$ (10).

RIA procedures. The umbilical cord plasma specimens were evaluated for concentrations of 17α-hydroxyprogesterone, 4-androstenedione, and testosterone both by our ID/GC-MS method and commercially available direct RIA procedures. The latter were used without and with a previous ether extraction procedure. The direct RIA assays were performed on plasma aliquots of 200 μL as described by the manufacturers using the [125]I-17α-hydroxyprogesterone RIA kit from ICN Biomedicals (Costa Mesa, CA), the ^{125}I-4-androstenedione RIA from Diagnostic System Laboratories (Webster, TX), and the ^{125}I-testosterone RIA kit from Diagnostic Products Corporation (Bad Nauheim, FRG). Regarding the diethyl ether extraction, 200 μL of plasma were extracted with 5 mL of diethyl ether by shaking vigorously for 5 min. After freezing the aqueous phase, the supernatant was decanted and evaporated under flowing nitrogen. For assay, the sample was reconstituted in 0.5 mL of PBS.

RESULTS

Application of our assay in a clinical setting is demonstrated by two examples of typical ion chromatograms: Figure 1 shows the steroid profile of an infant in whom 21-hydroxylase deficiency could be excluded. In another patient, a newborn, elevated 17α-hydroxyprogesterone could confirm 21-hydroxylase deficiency (Fig. 2).

The concentrations of plasma 17α-hydroxyprogesterone, 4-androstenedione, and testosterone determined by ID/GC-MS in normal subjects of various age groups are summarized in Tables 1–3.

17α-Hydroxyprogesterone, 4-androstenedione, and testosterone were assayed by ID/GC-MS and direct RIA with and without ether extraction in 20 samples of cord plasma. A comparison between the results produced by the different techniques is presented in Figure 3. The steroid values produced by ID/GC-MS were the lowest with the narrowest range. Direct RIA led to higher and more scattering steroid values. Ether extraction led to less scattering for all three hormones analyzed. A decrease in absolute steroid levels after ether extraction was noted only for 4-androstenedione and testosterone, whereas the mean levels of 17α-hydroxyprogesterone remained practically unchanged.

DISCUSSION

Currently, analytical methods based on mass spectrometry present the most specific quantitative methods for steroid determination (11, 12). In contrast to immunoassays, a physicochemical method such as ID/GC-MS is independent of phenomena such as cross-reactivity or matrix effects. Stable isotope-labeled analogs (13) are ideally suited as internal standards because they show similar chemical behavior and offer the advantage of compensation for losses of analyte during the workup procedure. They further avoid radioactive contamination of personnel and instrumentation. Gas chromatography bears the greatest potential in determining a whole spectrum of steroid hormones simultaneously in a single "profile," whereas RIA allow only the measurement of a single steroid at a time.

For the first time, basal plasma levels of 17α-hydroxyprogesterone, 4-androstenedione, and testosterone have been profiled by ID/GC-MS in normal female and male subjects at
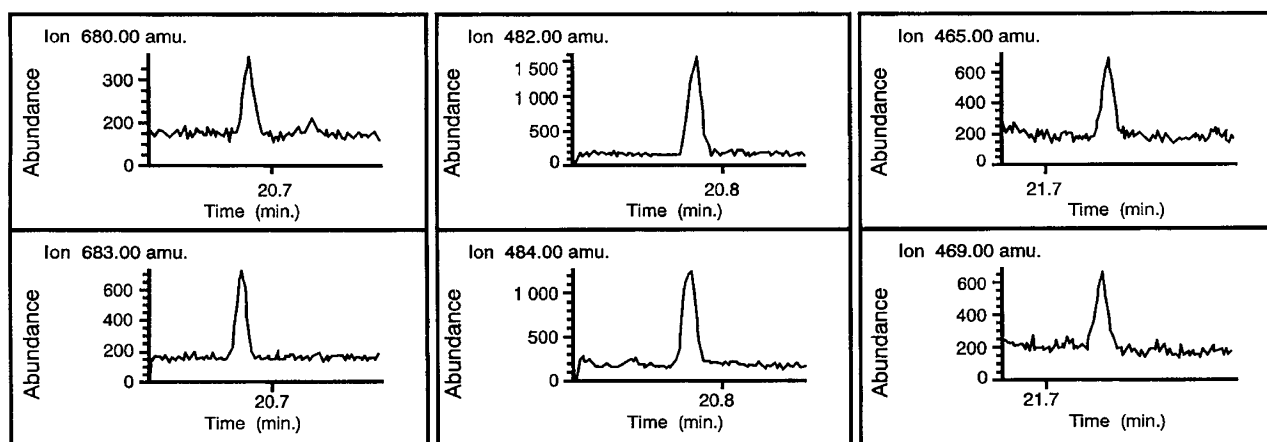

Figure 1. Exclusion of 21-hydroxylase deficiency in a 2-mo-old boy. Selected ion recording of a 0.1 aliquot of a processed extract of 0.5 mL of plasma. Each box contains the ion traces of typical corresponding fragments of a particular analyte (*upper half*) and its internal standard (*lower half*). Testosterone (m/z 680, m/z 683): 3.74 nmol/L; 4-androstenedione (m/z 482, m/z 484): 4.19 nmol/L; 17α-hydroxyprogesterone (m/z 465, m/z 469): 2.42 nmol/L.
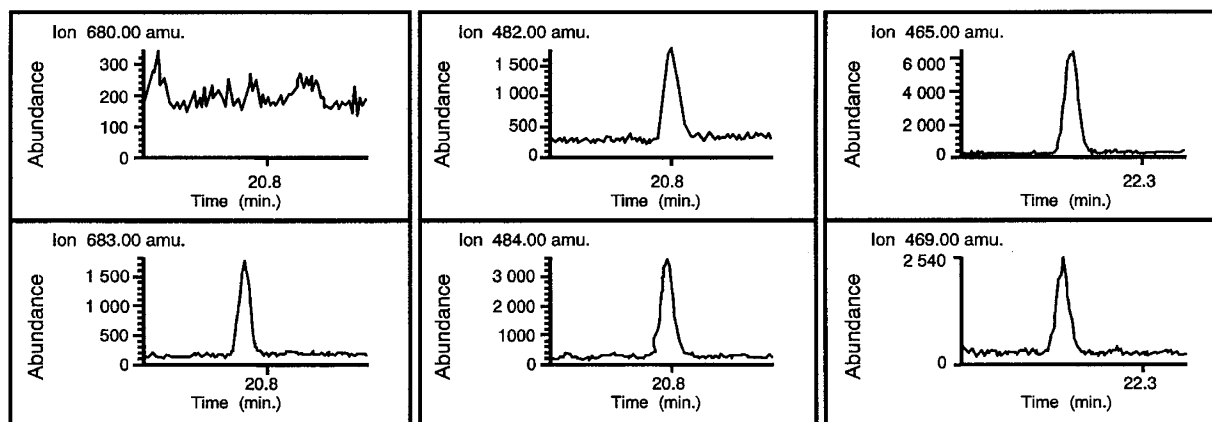
Figure 2. Diagnosis of 21-hydroxylase deficiency in a 3-d-old female patient with virilized genitalia. Selected ion recording of a 0.1 aliquot of a processed extract of 0.1 mL of plasma. Testosterone (m/z 680, m/z 683) not detected; 4-androstenedione (m/z 482, m/z 484): 15.65 nmol/L; 17α-hydroxyprogesterone (m/z 465, m/z 469): 66.81 nmol/L.

Table 1. *Concentrations of plasma 17α-hydroxyprogesterone (nmol/L) determined by ID/GC-MS in normal female and male subjects*

Age	Female subjects			Male subjects		
	n	Mean ± SD	Range	*n*	Mean ± SD	Range
Cord plasma	10	15.34 ± 11.83	6.74–43.69	10	15.97 ± 7.98	5.65–32.13
1–4 wk	6	2.11 ± 0.99	1.21–4.08	8	3.23 ± 1.48	1.27–6.20
2–12 mo	4	1.75 ± 1.87	<d.l.*–2.48	4	2.14 ± 0.66	1.42–2.90
2–6 y	7	0.48 ± 0.30	0.15–0.96	7	0.27 ± 0.15	0.18–0.54
7–10 y	9	0.75 ± 0.60	0.36–1.99	8	0.75 ± 0.57	0.39–1.66
11–15 y	9	1.27 ± 0.60	0.81–1.75	11	1.48 ± 0.69	0.42–2.29
>16 y	10	1.45 ± 0.75	0.69–2.54	10	3.29 ± 1.36	1.54–5.56

Note: To convert 17α-hydroxyprogesterone concentrations from nmol/L to ng/dL, multiply by 33.1.
* <d.l., below limit of detection.

Table 2. *Concentrations of plasma 4-androstenedione (nmol/L) determined by ID/GC-MS in normal female and male subjects*

Age	Female subjects			Male subjects		
	n	Mean ± SD	Range	*n*	Mean ± SD	Range
Cord plasma	10	7.58 ± 3.42	4.02–14.47	10	8.18 ± 1.18	5.13–9.30
1–4 wk	6	5.76 ± 1.60	1.22–6.25	8	3.91 ± 1.22	0.73–4.82
2–12 mo	4	2.37 ± 1.60	0.38–4.37	4	2.79 ± 1.15	0.97–3.84
2–6 y	7	0.70 ± 0.28	0.25–1.01	7	0.09 ± 0.04	0.04–0.15
7–10 y	9	1.08 ± 0.66	0.53–2.10	8	0.19 ± 0.08	0.13–0.32
11–15 y	9	4.16 ± 1.08	2.94–5.74	11	0.57 ± 0.35	0.29–1.39
>16 y	10	4.72 ± 2.65	2.24–10.49	10	1.13 ± 0.28	0.64–1.45

Note: To convert 4-androstenedione concentrations from nmol/L to ng/dL multiply by 28.6.

different ages. Our data inform about the actual hormonal concentrations and illustrate that commercial direct RIA can substantially overestimate the true steroid values.

Regarding 17α-hydroxyprogesterone, the most striking differences between our values and those determined by immunologic methods were found in cord plasma. Widely divergent plasma levels have so far been published (14–20). Using direct RIA, values as high as 203 ± 37.8 nmol/L (mean ± SD) in boys and 184 ± 34.5 nmol/L in girls have been reported (16). Values of comparable order were obtained using our in-house direct RIA (Fig. 3). RIA procedures using an additional purification step such as Celite chromatography led to much lower values: 92.7 ± 40.9 nmol/L in female subjects and 56.4 ± 30.7 nmol/L in male subjects (14). The values determined by ID/GC-MS were even 4–6 times lower than the latter and did not

show any significant sex difference (Table 1). Concerning the other age groups, the 17α-hydroxyprogesterone concentrations determined by ID/GC-MS were generally up to 2 times lower than those determined by RIA techniques using a chromatographic step (14, 17, 21, 22).

After an intense debate on the reliability of 17α-hydroxyprogesterone determined by direct RIA in the neonatal period and early infancy (9, 23–27), it could meanwhile be demonstrated that the interference of steroid monosulfates from the fetal adrenal zone, mainly 17α-hydroxypregnenolone sulfate, can lead to false positively elevated 17α-hydroxyprogesterone levels (28). The production of steroid sulfates in the fetal adrenal zone lasts well into infancy (29).

Our determinations of 17α-hydroxyprogesterone in umbilical cord plasma by direct RIA showed that 17α-hydroxypro-

Table 3. *Concentrations of plasma testosterone (nmol/L) determined by ID/GC-MS in normal female and male subjects*

Age	Female subjects			Male subjects		
	n	Mean ± SD	Range	n	Mean ± SD	Range
Cord plasma	10	0.27 ± 0.17	0.10–0.65	10	0.93 ± 0.24	0.45–1.25
1–4 wk	6	<d.l.*		8	8.19 ± 7.77	0.55–20.06
2–12 mo	4	<d.l.		4	2.46 ± 1.59	<d.l.–4.61
2–6 y	7	0.14 ± 0.04	0.07–0.28	7	0.18 ± 0.04	0.04–0.24
7–10 y	9	0.45 ± 0.17	0.21–0.66	8	0.28 ± 0.14	0.10–0.59
11–15 y	9	0.80 ± 0.10	0.69–0.97	11	7.15 ± 6.49	0.24–16.35
>16 y	10	0.94 ± 0.10	0.31–2.26	10	17.74 ± 4.27	13.26–26.04

Note: To convert testosterone concentrations from nmol/L to ng/dL, multiply by 28.8.
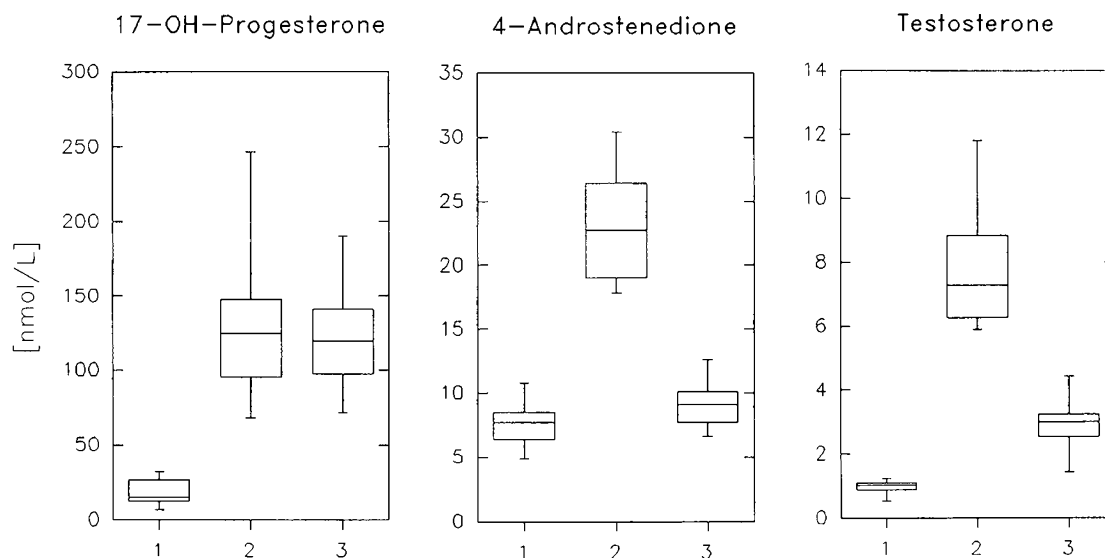* <d.l., below limit of detection.

Figure 3. Determination of 17-hydroxyprogesterone, 4-androstenedione, and testosterone in cord plasma from 10 male and 10 female neonates by ID/GC-MS (*1*), direct RIA (*2*), and direct RIA after ether extraction (*3*). Boxes mark 25th, 50th, 75th, and end of bars 10th and 90th centiles.

gesterone values hardly decreased after ether extraction (Fig. 3). We conclude that an additional extraction step does not necessarily improve the diagnostic efficiency of a direct assay. As mentioned before, even inclusion of chromatographic steps can obviously not eliminate all interferences in neonatal samples. These findings bear importance regarding screening programs for 21-hydroxylase deficiency, where, for reasons of practicability, direct immunologic procedures are advantageous but are likely to produce false positively elevated results.

In the case of 4-androstenedione, ID/GC-MS could not confirm the concentrations found in cord plasma using specific RIA with Celite chromatography (30). Inexplicably, the values found by ID/GC-MS (Table 2) were 2–3 times higher than the RIA values (female subjects, 3.3 ± 1.30 nmol/L; male subjects, 3.0 ± 0.98 nmol/L). In our experiments, direct RIA led to an overestimation of 4-androstenedione in cord blood (Fig. 3). This tendency was less expressed as in the case of 17α-hydroxyprogesterone. The interfering factor still remains to be determined. Unlike 17α-hydroxyprogesterone, ether extraction could reduce almost all interferences. In later stages of life, no substantial differences to other series were evident (22, 30).

Concerning testosterone in cord plasma, the values produced by ID/GC-MS showed a much more marked sex difference than has been previously assumed (30). Again, analysis by direct RIA resulted in marked overestimation, which partially

could be reduced after ether extraction (Fig. 3). In the neonatal period, our values for males corresponded with those that have already been published (30). In female neonates, however, testosterone was undetectable by our method. Concerning later life, no gross differences to other series could be found (22, 30).

The problems of lacking specificity and consecutive overestimation of true steroid values by immunologic methods, especially direct assays, is of particular importance not only in the newborn period and early infancy. Problems with steroid immunoassays have further been reported in prepuberty (3), in female samples (4, 5, 7), in pregnancy (4), in disorders of steroidogenesis (6, 31, 32), or when analyzing different media, *e.g.* ovarian follicular fluid (2). ID/GC-MS could help circumvent these analytical problems. If the technique was used in different studies, comparability between data would not present a problem because no individual reference ranges would be needed for each immunoassay (6).

The potential of ID/GC-MS in quantitative plasma steroid analysis has not yet been exploited to its full extent. So far, ID/GC-MS has found application as a reference methodology presenting the "gold standard" for the evaluation of steroid immunoassays (12). Its use as a routine method has been lacking, however. Drawbacks concerning the use of ID/GC-MS in a clinical setting have primarily been the huge costs

of the sophisticated instrumentation, complex workup procedures, and the unavailability of most stable isotope-labeled internal standards. However, during the last decade, vast technical improvements have rendered the development of reliable labor and cost effective GC/MS instruments possible. With respect to appropriate internal standards, several suitable pathways leading to nonradioactive internal standards have meanwhile been published (13).

We have recently developed an ID/GC-MS method for the profiling of 17α-hydroxyprogesterone, 4-androstenedione, and testosterone which we believe to be clinically applicable. Our method allows "day-return" reporting of results. Sample preparation is simple and can be completed within 6 h. Up to 20 specimens can conveniently be prepared in parallel, and up to 20 samples can be analyzed per day on a single GC/MS instrument (10). Of course, regarding the possible number of samples to be analyzed, GC/MS cannot compete with direct immunoassays because the latter techniques require only minimal sample preparation and allow analysis of numerous samples in batch assays in contrast to only serial assays possible with GC/MS.

We therefore suggest to set up priorities for steroid analysis by ID/GC-MS. Thus, a small number of specialized laboratories equipped with the analytical instrumentation and expertise should suffice. Our ID/GC-MS assay could be applied to the analysis of plasma from neonates or young infants suspected of 21-hydroxylase deficiency. Another field of application would be the prenatal diagnosis of 21-hydroxylase deficiency from amniotic fluid. Simultaneous measurement of 17α-hydroxyprogesterone, 4-androstenedione, and testosterone in amniotic fluid is advantageous in detecting the sex of fetuses (33) and all cases of 21-hydroxylase deficiency (34). We recently demonstrated (35) that reliable steroid analysis still remains a pillar of pre- and postnatal diagnosis of 21-hydroxylase deficiency in case no index patient is available.

To conclude, plasma steroid analysis by ID/GC-MS will not replace immunoassays in general. Besides its role as a reference methodology, we suggest application of ID/GC-MS in a clinical setting whenever problems from matrix effects or cross-reactivity are likely to arise or suspicious results by immunoassays need to be rechecked.

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